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Immunological biomarkers as indicators for outcome after discontinuation of nucleos(t)ide analogue therapy in patients with HBeAg negative chronic hepatitis B

Running title: Biomarkers for cessation of NAs in CHB

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ABSTRACT

Background and Aims

The optimal duration of treatment with nucleos(t)ide analogues (NAs) for patients with HBeAg-negative Chronic Hepatitis B (CHB) is unknown. The aim of this study was to identify an immune signature associated with off-treatment remission to NA therapy.

Methods

We performed microarray analysis of PBMCs from six patients with chronic hepatitis B who stopped NA therapy (3 with off-treatment remission, 3 with relapse) and 5 patients with chronic HBV infection (previously termed “inactive carriers”) served as controls. Results were validated using qRT-PCR on a second group of 21 individuals (17 patients who stopped treatment and 4 controls). PBMCs from 38 patients on long-term NA treatment were analysed for potential to stop treatment.

Results

Microarray analysis indicated that patients with off-treatment remission segregated as a distinct out-group. Twenty-one genes were selected for subsequent validation. Ten of these were expressed at significantly lower levels in the patients with off-treatment remission compared to the patients with relapse and predicted remission with AUC of 0.78-0.92. IFN γ , IL-8, FASLG and CCL4 were the most significant by logistic regression. Twelve (31.6%) of 38 patients on long-term NA therapy had expression levels of all these four genes below cut-off values, and hence were candidates for stopping treatment.

Conclusions

Our data suggest that patients with HBeAg-negative CHB who remain in off-treatment remission 3 years after NA cessation have a distinct immune signature and

that PBMC RNA levels of IFN γ , IL-8, FASLG and CCL4 may serve as potential biomarkers for stopping NA therapy.

Keywords: Chronic hepatitis B; Nucleos(t)ide Analogues; treatment discontinuation

INTRODUCTION

Chronic infection with hepatitis B virus (HBV) remains a serious public health problem worldwide with a high morbidity and mortality rate ¹. Patients with active viral replication and necro-inflammation who remain untreated are at particular risk for cirrhosis and hepatocellular carcinoma (HCC) ^{2, 3}. The infection can be controlled either with a limited course of interferon- α or long term nucleos(t)ide analogue (NA) therapy ^{4, 5}. Potent first line nucleos(t)ide analogues (NAs) have been widely used and found to be associated with virological response, biochemical remission, reversion in liver fibrosis and significant reduction in morbidity and mortality ⁵⁻⁸. However, long-term administration of NAs has raised several safety and economic issues, making the question of treatment discontinuation an emerging issue especially in patients with HBeAg negative chronic hepatitis B (CHB). Several small studies have shown that a proportion of patients remained in remission after NA discontinuation ⁹⁻¹³. Therefore, the Asian Pacific Association ¹⁴ and more recently European guidelines ⁵ recommended that NAs could be discontinued in selected, non-cirrhotic HBeAg negative patients who have achieved virological suppression for at least 3 years and are under close monitoring ⁵. However, as 55% to 70% of patients relapse within 24 months after NA discontinuation, and since no acceptable end point marker exists to safely predict long term off treatment remission, NA discontinuation in HBeAg negative patients still remains a debatable issue ⁹⁻¹³.

Control of HBV infection involves the host immune response to control viral replication, whilst HBV has evolved mechanisms to evade both innate and adaptive immune responses in order to establish persistent infection ¹⁵. Long-term therapy with NAs seems to facilitate the restoration of the host immune system following reduction of serum HBV DNA ¹⁶. Biochemical rebound in patients after NA

discontinuation, followed by HBsAg clearance in some of them, might be explained by the restoration of immune system functions at the time of NA discontinuation. Patients who can control virological rebound and remain in remission or develop off-treatment HBsAg clearance may have different immunological characteristics compared to individuals in different stages of HBV infection. In this study we investigated whether peripheral blood mononuclear cell (PBMC) RNA levels are associated with off-therapy remission acting therefore as potential immune-related biomarkers to identify individuals with HBeAg-negative CHB on NA therapy who can benefit from stopping treatment.

PATIENTS AND METHODS

Patients

In July 2013 we initiated a prospective multicentre study examining the potential of NA discontinuation in patients with HBeAg-negative CHB who were receiving NA therapy for at least five years and had no evidence of cirrhosis before NA initiation. The current study represents a group of patients with long-term (3 years) of follow-up after treatment discontinuation that were recruited from the outpatient clinic of the 2nd Academic Department of Internal Medicine at “Hippocraton” General Hospital of Athens, Greece.

Twenty-three adult patients under maintenance therapy stopped NA treatment. The main inclusion criteria for participation in the discontinuation study were as follows: documented HBeAg negative chronic hepatitis B before NA therapy initiation, absence of cirrhosis defined by liver stiffness <10 kPa assessed by transient elastography (Echosens) or Ishak score <4 at liver biopsy; treatment with NA or a

combination of NAs for at least 5 years and undetectable serum HBV DNA for at least 4 years. Patients were excluded if they had: co-infection with hepatitis D virus (HDV), hepatitis C virus (HCV) or human immunodeficiency virus (HIV); any additional cause for liver injury; history of liver decompensation, malignancy including hepatocellular carcinoma (HCC) or history of liver transplantation. The study was approved by an independent ethics committee of the Hippocraton General Hospital and was conducted according with the principles of the Declaration of Helsinki. All patients signed an informed consent before screening for their participation.

Nine untreated patients with HBeAg-negative chronic HBV infection (previously termed “inactive carriers”) served as controls for the present study. All of them were HBsAg positive, HBeAg negative / anti-HBe positive with ALT values <40 IU/l on at least 4 occasions for the last 12 months, serum HBV DNA levels <2,000 IU/mL and liver stiffness < 7 kPa at transient elastography.

A second matched population of thirty-eight non-cirrhotic HBeAg-negative CHB patients under maintenance therapy without stopping NAs were recruited to assess the potential for using cut-off values of identified genes to stop treatment.

Study Design - Definitions

The study was divided into two parts. Firstly, an investigative study to identify determinants associated with off-treatment remission and secondly a cross-sectional analysis of patients on long-term treatment (Figure 1). This second part was conducted to investigate if determinants identified in the first part of the study could be used to subdivide patients on NA therapy into two discrete populations, one of which could be targeted for cessation of therapy.

PBMC samples for gene expression analysis were collected at the end of treatment (EOT) and at 6th months after NA discontinuation for three of the patients. All patients were followed-up for at least 36 months after stopping treatment. According to the protocol all patients had monthly visits during the first 3 months and every 2 to 3 months thereafter. Clinical examination and liver function tests were performed at each visit. Serum HBV DNA levels were measured every 3 months for the first year and 6 monthly thereafter. Serum HBsAg quantification was performed at the EOT and every 6 months. The upper limit of normal (ULN) for ALT and AST was 40 IU/L and serum HBV DNA levels were assessed by a polymerase chain reaction assay with sensitivity of 13 IU/mL.

In the present study the definition of remission was based on the combination of biochemical and virological profile; remission was defined as ALT < 40IU/l and serum HBV DNA levels < 20,000 IU/mL or ALT > 40IU/l in only one-measurement and serum HBV DNA levels < 20,000 IU/mL for at least 36 months after treatment cessation.

Methods

Isolation of PBMCs and mRNA purification

Blood samples were collected from 23 patients who stopped NA treatment at the End Of Treatment (EOT) and 3 of the patients with off-treatment remission at 6th months post-therapy cessation. Blood samples were also collected from 38 patients on long-term NA therapy and from 9 controls. PBMCs were isolated from 10-20 mls blood using Ficoll Histopaque 1077-1 (SIGMA, St Louis, MO, USA) density centrifugation. Cell pellets were lysed with TRIzol (Invitrogen, Life Technologies, Grand Island, NY, USA) and kept at -80 °C until mRNA extraction. mRNA was extracted and purified

using the RNeasy Mini kit (QIAGEN, CA, USA). A NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware) was used to determine the quality and quantity of the mRNA.

Agilent Whole Human Genome Oligo Microarrays

Agilent Whole Human Genome Oligo Microarrays (Miltényi Biotec, GmbH) were used to analyse PBMCs: taken at the EOT from 3 patients with off-treatment relapse; from 3 patients with off-treatment remission taken at the EOT and at 6th months post-treatment; and from 5 controls. Microarray image files were processed using Agilent Feature Extraction software (FES, Agilent Technologies, Inc). For determination of differential gene expression FES derived output data files were further analysed using the Rosetta Resolver® Gene expression data analysis system (Rosetta Inpharmatics LLC). The signal intensities were normalized by dividing the intensity values by their median. The significance threshold was a nominal p-value $p < 0.05$. Principal component analysis (PCA) was performed using the rgl package in R¹⁷. Results were also displayed as a Heat map using MultiExperiment Viewer¹⁸. To assess the relationship between the genes, Kyoto Encyclopaedia of Genes (KEGG) and Genomes pathway analysis (DAVID v6.7)¹⁹ software were applied.

Reverse transcription - qRT-PCR

mRNA samples of 10 patients with off-treatment relapse (at the EOT), from 7 patients with off-treatment remission (at the EOT), from 4 controls and from 38 patients on NA therapy were used for qRT-PCR. cDNA was reverse transcribed using Applied Biosystems High Capacity cDNA Kit (USA). qPCR was performed using Taqman® Low Density Array System (Life Technologies, USA). The TaqMan array was

designed for custom configuration with 21 pre-dispensed primer assays for the target genes and 3 for the endogenous controls (Housekeeping genes: GAPDH, B₂-microglobulin and B-actin). Relative levels of gene expression were calculated using ΔCt (threshold cycle) method. The GAPDH housekeeping gene was used to normalize the RNA amounts. Results are expressed relative to GAPDH using the equation: Relative quantity (Fold change): $2^{-\Delta\text{Ct}}$, where $\Delta\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{GAPDH}}$

Statistical Analysis

Data was analysed using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA) and SPSS (version 22; SPSS Inc, Chicago, IL). Patient characteristics were compared using Mann-Whitney test for continuous variables and chi-square or Fisher's exact tests for categorical variables. The Mann-Whitney test was used to compare gene expression levels between the clinical groups and Wilcoxon matched-pairs signed rank test to compare gene expression levels between different time points of the same individuals. Receiver Operating Characteristic (ROC) curves for the accuracy of prediction of off-treatment remission or relapse were derived and thresholds of gene expression levels were calculated [with areas under the ROC curves (AUC_{ROC}) and their 95% confidence intervals (CI)]. Gene expression levels were log-transformed to achieve normality and to be included in the univariable logistic regression analysis. Multivariable logistic regression analysis was applied to the predictors, which were significant at the univariable analysis. Correlations were analysed using the Spearman's rank test (ρ). The level of $p < 0.05$ was considered to be statistically significant.

RESULTS

Population characteristics and outcome

The main characteristics of the patients are shown in Table 1. For the individuals that stopped NA therapy, the mean age was 59 ± 9 years and 15 of the 23 patients were males. The median duration of NA treatment was 8 (5-14) years and the median off-treatment follow-up period 4.6 (3.2-5.2) years. Off-treatment remission was observed in 10 of the 23 patients (43%); Seven out of ten patients with remission achieved non-detectable serum HBV DNA levels at 30 (± 20) months after treatment discontinuation. Two patients had ALT >40 U/mL in only one measurement during the follow-up.

According to the study design, six of the 23 patients and 5 of the controls were used to perform whole genome gene expression analysis (“development cohort”). Three out of six patients relapsed after a mean of $2.6 (\pm 0.6)$ months and three had off-treatment remission with median serum HBV DNA levels 172 (0-9300) IU/ml, 2000 (0-4000) IU/ml, 2470 (0-4410) IU/ml at 12, 24 and 36 months respectively. Seventeen of the 23 patients and four of the controls were studied as a “validation cohort” performing the qRT-PCR gene expression analysis. Ten of these had off-treatment relapse after a mean of 5.2 ± 4 months and seven had remission with median serum HBV DNA levels 3084 (0-9200) IU/ml, 170 (0-6650) IU/ml, 63 (0-10907) IU/ml at 12, 24 and 36 months respectively. HBV DNA levels of the patients with off-treatment remission during the follow-up period are shown in Supplementary Table 1. No significant difference in baseline characteristics was observed between the development and validation groups (Table 1).

Patients with off-treatment remission have a distinct gene expression profile

Micro array analysis was performed in “the development sub-cohort”: three patients with off-treatment relapse at the end of treatment (EOT); three patients with off-treatment remission at the EOT and six months following cessation of therapy; and five controls. This demonstrated that there were 1649 differentially expressed genes ($p < 0.05$ after Bonferoni correction) between patients with off-treatment remission and relapse, 2231 differentially expressed genes between patients with off-treatment remission and controls, and 730 differentially expressed genes between patients with off-treatment remission at the EOT and at 6 months post-treatment. One hundred and sixty differentially expressed genes had a greater than two-fold increase and 201 a greater than two-fold decrease between patients with off-treatment remission and relapse. Unsupervised hierarchical clustering analysis of the oligo-microarray data suggested that the three patients with off-treatment remission at the EOT clustered as a distinct group compared to the other groups (relapsers, controls and patients with remission at 6 months after treatment cessation) (Figure 2A), which was also illustrated by the principal component analysis (PCA) (Figure 2B).

The heat map, which was created using only the differentially expressed genes between patients with remission at the EOT and relapse, again demonstrated that the group with remission clustered as an out-group to the others (Figure 2C). The cytokine-cytokine receptor interaction pathway was the most significantly different in the off-treatment remission group compared to the other groups [$p = 3.14 \times 10^{-4}$, false discovery rate (FDR) 0.002] as determined by Kyoto Encyclopedia of Genes (KEGG) and Genomes pathway analysis (DAVID v6.7).

Comparison of expression levels for the target genes between patients with off-treatment remission and relapse.

We selected 21 differentially expressed key genes based on the fold change difference between the patients with off-treatment remission and relapse, their participation in the cytokine-cytokine receptor interaction pathway and their potential immunological relevance for controlling HBV infection. All had p values of less than 0.05 after Bonferoni correction. The levels of expression for these 21 target genes were determined by qRT-PCR on the “validation sub-cohort”. The results indicated that 10 of the 21 genes were expressed at significantly lower levels in the patients with off-treatment remission as compared to the patients with relapse (Figure 3). Specifically, patients with remission had lower expression of CCL20 (14 fold decrease (fd), $p=0.03$), CCL4 (5.9 fd, $p=0.02$), CXCL2 (18 fd, $p=0.02$), CXCL3 (17.6 fd, $p=0.01$), IFN γ (5.3 fd, $p=0.01$), IL-8 (5.7 fd, $p=0.01$), IL-1A (61 fd, $p=0.03$), IL-1B (8.6 fd, $p=0.05$), FASLG (2 fd, $p=0.01$) and TNFRSF9 (2.9 fd, $p=0.05$) (Supplementary Table 2). Additionally, ROC curve analysis of the 10 target genes showed that expression levels of the genes was a potentially useful marker for discriminating patients who will have off-treatment remission with AUROCs of 0.78-0.92 (95% CI: 0.56-1.00, $p < 0.05$) (Figure 4). The best performing genes were CCL4 (AUROC: 0.929), IFN γ and IL-8 (AUROC: 0.871), CXCL3 and FASLG (AUROC: 0.857).

Predictive model of the off-treatment remission or relapse

Univariable logistic regression analysis of the expression of the 10 target genes indicated that low expression of the target genes was associated with off-treatment remission after NA therapy cessation (Table 2). Specifically, we observed significant

association for FASLG ($p=0.030$), IFN γ ($p=0.032$), IL-8 ($p=0.048$) after log-transformation and a trend for the CCL4 ($p=0.053$). Multivariate logistic regression analysis revealed that none of the four genes associated with off-treatment remission on univariate analysis remained independently significant; a trend towards association with off-treatment remission was observed for IFN γ ($p=0.073$).

Expression levels of the target genes and HBsAg clearance

Four of the twenty-three patients (17.4%) who stopped NA therapy achieved HBsAg loss after a mean of 30 (± 23) months. These patients had a mean age 71 ± 4 years, ALT levels 20 ± 3 IU/mL and liver stiffness 5.4 ± 1 kPa. The duration of NA treatment was 6 ± 0.5 years, 3 were treated with TDF and one with ETV. The duration of follow-up for them was 54 ± 12 months and at EOT all patients had HBsAg < 1000 IU/mL with two having levels < 100 IU/mL. These four patients had significantly lower expression levels of FASLG ($p=0.04$), IL-8 ($p=0.02$), CCL4 ($p=0.008$) and a trend for IFN γ ($p=0.06$), compared to those who did not achieve seroclearance during follow-up.

Stratifying patients on NA treatment using levels of immune response genes

Expression of CCL4, IFN γ , IL-8 and FASLG, the four genes that were significantly associated with off treatment remission in the logistic regression analysis, was determined by qRT-PCR in the 38 patients on long-term NA therapy to determine if a discrete sub-group of individuals could be identified with a similar immune signature to the sustained responders. These individuals could then be potential candidates for stopping treatment. We used ROC curve analyses of the validation sub-cohort to derive the cut-off values in gene expression levels, which discriminate patients into two sub-groups. Patients with gene expression levels lower than the cut-off values

will have potential off treatment remission and higher potential relapse. We applied these cut-off values on the gene expression levels of the 38 on treatment patients. Patients with CCL4 expression levels lower than 0.098, IFN γ expression levels lower than 0.0057, IL-8 expression levels lower than 0.12 or FASLG expression levels lower than 0.0038 could be potential sustained responders with a sensitivity of prediction 71.4-85.7% and specificity 80-90% (Figure 5A). Twelve (31.6%) of the 38 patients had expression levels lower than the cut-off values for all four genes and therefore could be considered for stopping treatment (Figure 5B).

DISCUSSION

NAs are widely used by the majority of the physicians in the treatment of patients with CHB and they have been proved very effective and safe ^{4, 5}. Long term maintenance therapy with NAs is associated with sustained viral suppression and an improvement in histology and overall outcomes ⁶⁻⁸. However, it does not affect serum HBsAg levels and HBsAg loss rarely is achieved particularly in HBeAg negative patients ^{20, 21}. Recently, a number of small studies have shown that NA therapy can be stopped in patients with HBeAg negative CHB who have long-term viral suppression and the evaluation of NA treatment withdrawal has shown higher HBsAg loss rates than in studies with long-term NA. This has resulted in inclusion of possible treatment withdrawal as an option in recent European guidelines for the treatment of HBV infection, based on the duration of HBV DNA negativity ⁵. The host immune response plays a crucial role in HBV clearance and is expected to participate in the maintenance of an off-treatment remission ²²⁻²⁴. This is the first study investigating PBMC RNA levels as an accessible indicator of immune activity in patients with HBeAg negative CHB who stopped long term NA therapy. Our results have shown that low gene expression of CCL4, IFN γ , IL-8 and FASLG is associated with off treatment remission.

Our study was based on prospectively collected data from a clinical multicenter cohort of HBeAg negative non-cirrhotic patients. We used two different cohorts. In the development cohort we analyzed PBMCs using the whole genome microarray methodology. We found that patients with off-treatment remission had a distinct immunological pattern, which was confirmed when we performed qRT-PCR in the validation cohort. Ten out of 21 genes were significantly down regulated in the patients with off-treatment remission as compared to the patients who relapsed

during the follow up. These genes belong to the cytokine-cytokine receptor interaction pathway, are pleiotropic and secreted by both adaptive and innate immune cells. Using robust statistical analysis with the areas under the curve (AUC) and logistic regression we found that four genes (IFN γ , IL-8, FASLG and CCL4) could serve as predictive factors for off-treatment remission with AUC: 0.78-0.92 (95% confidence interval (CI) 0.56 to 1, $p < 0.05$). None of these factors are independently associated with off-treatment remission, which is not surprising as the immune system is complex with multiple interactions and individual immune components do not function in isolation. The same four genes, which predict off-treatment remission, are down regulated in the patients with successful seroclearance during the follow-up supporting again the immunological potency for sustained HBV suppression after NA discontinuation.

We hypothesized that gene expression patterns within the PBMC would indicate if there was a beneficial immune status that was predictive of remission. Overall this appear to show that lower levels of markers associated with immune cell activation were found in the patients with off-treatment remission, suggesting that their immune system had been “turned down”. Patients with off-treatment remission had a distinct immunological pattern at the EOT compared to 6 months post-treatment probably due to a subsequent increase in viral load with an associated immune response. One hypothesis is that the turning down of the immune system may subsequently result in “re-priming” of the immune system if the virus rebounds, and resulting in better subsequent viral control, as observed by Höner zu Siederdissen et al, who correlated rebound in HBV DNA with subsequent HBsAg loss ²⁵. Further longitudinal studies would help to ascertain if these individuals relapse in the long run and if this is associated with an up-regulation of these immune markers. IFN γ is a well-known

effector cytokine, an immune marker, which can “turn on” the cellular antiviral mechanism by inducing the expression of several antiviral proteins modulating both innate and adaptive immune responses ^{26, 27}. FasL and IL-8 have both been previously associated with active CHB. In particular, the HBx protein can transactivate the FasL ^{28, 29} and the IL-8 promoters ³⁰, thereby up regulating the expression of these genes. Also, HBV-specific T cells can produce IL-8 during active CHB, and flares of liver inflammation (either spontaneous or induced by anti-viral withdrawal) have been shown to be preceded by a parallel increase of IL-8 production and serum HBV DNA levels ³¹⁻³⁴. Specific inhibition of IL-8 increased the potency of IFN α against HBV *in vitro* and the addition of recombinant human IL-8 almost totally rescued HBV replication during IFN challenge, strongly suggesting that IL-8 expression induced by HBV can impair the ability of endogenous IFN α to inhibit early stages of viral replication, thus favoring viral persistence ³⁵. Inhibition of IL-8 activity could be instrumental to the suppression of viral activity and to the improvement of IFN α antiviral action.

Previous work from Rivino et al ³⁶ and Rinker et al ³⁷ have analysed T cell responses following cessation of NA therapy. Both studies used *in vitro* stimulation of T cells with HBV peptides in order to specifically amplify low frequency T cell responses, with contrasting results. In the study of Rivino et al, expression of PD-1 was associated with beneficial responses as defined by an absence of a flare in ALT, whereas Rinker et al described an absence of PD-1 on T cells as being beneficial in terms of HBsAg loss. These data are consistent with discrete mechanisms operating in these two different scenarios. For prolonged viral suppression, the presence of PD-1 positive T cells, which may represent T cell memory populations with improved survival characteristics ³⁸, appears important. Whereas following a virological

relapse T cell priming may be required, in which case PD-1 negative T cells may be more important. Our data studies patients with a response pattern similar to the work of Rivino, in which memory responses may be present and so lower levels of T cell help are required to maintain immune response, and control of viral replication. Thus the immune response appears to be turned down. Furthermore, low frequency T cell responses, which require antigen specific stimulation *in vitro* in order to become detectable, are unlikely to be identified using our unbiased approach.

The percentage of our patients with off-treatment remission and HBsAg loss is in accordance with the results of other clinical studies^{9, 10, 13, 39-41}. Although several studies tried to explore the factors that may predict the post-cessation outcome, none revealed a strong or reliable indicator^{10, 13, 25, 39, 42, 43}. The significance of serum HBsAg levels at the time of discontinuation as an indicator of remission has been debatable with contradictory results among the studies^{13, 41, 44-48} while novel HBV markers, such as core-related antigen (HBcrAg)^{49, 50} and HBV RNA^{51, 52} need to be assessed in larger cohorts prospectively. The data regarding the course of patients before losing HBsAg after NA discontinuation are conflicting¹¹. Interestingly, Höner zu Siederdissen et al²⁵ reported that patients with HBsAg loss had significant increases in serum HBV DNA after treatment discontinuation. These authors also reported the presence of antigen-specific PD-1 negative T cells correlating with this³⁷. As noted above this population appears to be immunologically distinct from individuals who have prolonged viral suppression. However given the conflicting results in this area, we consider that an approach which predicts prolonged viral suppression would currently be more clinically applicable, than one which relies on a later association with HBsAg loss. Further studies in this area will be important both in identifying additional markers, but also in determining which short term outcomes are best

predictive of long term benefit.

The study has some limitations. Applying immunological predictive factors in clinical practice is more cumbersome than serological analysis, however given current technological advances and the advent of personalized medicine, costs are likely to fall. Additionally, further investigation is needed to define the thresholds of gene expression levels giving the highest AUC, which may predict the off-treatment remission. The small number of patients who stopped NA treatment is another drawback but the risk of post-treatment relapse and the need for close follow-up after treatment cessation made the identification of these patients challenging. Thirdly, we included non-cirrhotics, Caucasians with HBV genotype D infection patients and therefore our results may not be generalizable to all patients. Finally, it might be argued that off-treatment remission is a dynamic condition and remission or relapse rates may change during the follow up with the percentages of relapse varying considerably among studies ¹⁰. However, we should emphasize that we have prospectively collected data, we used strict criteria for remission and all patients have been followed for 36 months, which strengthen our data.

In conclusion, our results demonstrated that the HBeAg-negative CHB patients on effective long-term therapy with NAs who stopped NA treatment and had off-treatment remission seem to share a distinct immune profile from those patients with an off-treatment relapse. Expression levels of the CCL4, IL-8, IFN γ and FASLG genes could be used as potential biomarkers of off-treatment remission and could stratify those patients on NA treatment for consideration of treatment cessation. Our data showed for first time the predictive significance of immunological biomarkers in patients with HBeAg-negative CHB who stopped NAs and underlined the need for further research with a higher number of patients confirming our results.

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TABLES

Table 1: Baseline characteristics of the chronic hepatitis B (CHB) patients and inactive carriers (controls). No significant differences were observed between the patients or controls

Baseline characteristics	Development cohort (N=11)		Validation cohort (N=21)		Cross-sectional cohort
	CHB Patients	Inactive carriers (Controls)	CHB Patients	Inactive carriers (Controls)	CHB Patients
Number (N)	6	5	17	4	38
Gender (M/F)	3/3	2/2	12/5	2/3	26/12
Age (years)†	57±8.4	44±19	59.8±11	37.5±10.4	53.9±12
ALT (IU/ml)†	23.2±5.6	23±3.4	22.4±6.2	21±5.4	22±7.6
HBV DNA (IU/ml)†	Undetectable	864±1019	Undetectable	1091±425	Undetectable
HBsAg (log ₁₀ IU/ml)†	3.4±3.4	N/A	3±3.1	N/A	N/A
Duration of NA treatment (years)‡	8 (6-14)	0	8 (5-13)	0	8 (5-13)
Duration of follow-up (months)‡	65 (61-68)	-	61 (38-68)	-	-

(†Mean ± SD, ‡Median (range), NA: Nucleos(t)ide Analogue, N/A: Not available)

Table 2: Univariable logistic regression analysis of the expression of the 10 target genes with the off-treatment remission and relapse.

Variables	B	S.E	P value	Odds Ratio	95% CI of OR	
					Lower	Upper
CCL20	0.653	0.344	0.057	1.922	0.980	3.769
CCL4*	3.317	1.911	0.053	27.568	0.652	1165.958
CXCL2	0.954	0.535	0.074	2.596	0.910	7.401
CXCL3	1.257	0.663	0.058	3.514	0.958	12.894
FASLG*	3.394	1.568	0.030	29.783	1.379	643.079
IFN γ *	1.242	0.580	0.032	3.463	1.112	10.788
IL-1A	0.618	0.332	0.063	1.855	0.968	3.555
IL-1B	0.858	0.484	0.076	2.358	0.914	6.083
IL-8*	1.090	0.550	0.048	2.973	1.012	8.737
TNFSF9	1.188	0.625	0.057	3.280	0.963	11.166

* $P \leq 0.05$

FIGURE LEGENDS

Figure 1: Flow-chart diagram illustrating study design

Patients: Non-cirrhotic HBeAg-negative Chronic Hepatitis B (CHB) patients on long-term treatment with Nucleos(t)ides analogues (NAs); Sub-cohorts: Patients who stopped NA therapy (Development and Validation cohort) and had at least 36 months of follow-up (n=23); Patients on NAs without cessation of therapy (Cross-sectional cohort, n=38); **Controls (C):** Nine untreated patients with HBeAg-negative chronic HBV infection (previously termed “inactive carriers”); **Off-treatment remission** was defined as ALT < 40IU/l and serum HBV DNA levels < 20,000 IU/mL or ALT > 40IU/l in only one-measurement and serum HBV DNA levels < 20,000 IU/mL for at least 36 months after treatment cessation

Figure 2:

(2A) Hierarchical clustering analysis for all the genes from the microarray analysis showing that patients with off-treatment remission (REM-EOT) cluster as an out-group; **(2B)** Principal component analysis for all the genes. **(2C)** Heat Map representing the microarrays comparing the differentially expressed genes between patients with off-treatment remission and relapse at the end of treatment (EOT). Genes which are relatively up-regulated are coloured red and those down-regulated are coloured green. REL-EOT (N=3): patients with off-treatment relapse (End Of Treatment), REM-EOT (N=3): patients with off-treatment remission (End Of Treatment), REM-6 months (N=3): patients with off-treatment remission (6th months off-treatment for the same patients as REM-EOT), Control (N=5): untreated inactive carriers

Figure 3: Relative mRNA expression of the 10 target genes, which are significantly differentially expressed between the patients with off-treatment remission (REM-

EOT, n=7), relapse off-treatment (REL-EOT, n=10) and untreated carriers with inactive disease (n=4). mRNA was quantified by qRT-PCR, expression levels normalised to glyceraldehyde phosphate dehydrogenase (GAPDH) and the result expressed as fold expression. Shown are the mean \pm SD expression levels. *p <0.05, **p \leq 0.01

Figure 4: Receiver Operator Characteristic (ROC) for prediction of off-treatment relapse using the expression levels of the 10 significantly differentially expressed genes. The Areas Under the ROC Curve (AUC) are shown.

Figure 5: (5A) CCL4, IFN γ , IL-8 and FASLG expression levels of the patients on treatment (n=38). The cut-off values as determined by the ROC analyses were used to divide the population into two subgroups, one with potential off-treatment remission (potential REM) and one with potential off-treatment relapse (potential REL). Bars represent mean \pm SD. **** p-value < 0.0001. **(5B)** The percentage of patients on treatment with one, two, three or four genes below the cut off values.